REMARKS

This is a full and timely response to the Office Action mailed February 22, 2007, submitted concurrently with a one month Extension of Time to extend the due date for response to June 22, 2007.

Claims 20 and 28 have been amended to more particularly define the present invention. Support for the claim amendments can be found throughout the specification and the original claims. Thus, claims 20-32 are currently pending in this application.

In view of this response, Applicants believe that all pending claims are in condition for allowance. Reexamination and reconsideration in light of the above amendments and the following remarks is respectfully requested.

Rejections under 35 U.S.C. §112

Claims 29-32 are rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement. Applicant respectfully traverses this rejection.

The Examiner has argued in the Office Action that the claims are not limited to the liposomal formulation taught and used in the specification or a formulation made in a similar manner. The Examiner has also stated that Applicant has not shown the protection of mice as disclosed and correlated to the features of the claimed methods. Applicants respectfully disagree with the Examiner's conclusions in this regard for the same reasons as that set forth in the responses filed March 6, 2006 and August 1, 2006 especially since the Examiner has not specifically responded to the points raised in the previously presented arguments.

Applicants believe that the specification clearly describes the manner and process of practicing the claimed invention in full, clear, concise and exact terms so as to enable one skilled in the art to make and use the claimed liposomal formulation *to the fullest extent of the claims*. In other words, one skilled in the art, based on the teachings of the specification and the knowledge in the art, can practice the *full scope* of the method of the present invention without undue experimentation. As stated in § 2164.01(a) of the Manual of Patent Examining Procedure (MPEP), there are many factors to consider when determining whether there is sufficient evidence to support

a conclusion that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is undue. These factors include, but are not limited to:

(1) the breadth of the claims,
(2) the nature of the invention,
(3) the state of the prior art,
(4) the level of one of ordinary skill,
(5) the level of predictability in the art,
(6) the amount of direction provided by the inventor,
(7) the existence of working examples, and
(8) the quantity of experimentation needed to make or use the invention based on the content of the disclosure

The Examiner's concerns appear to focus on the breadth of the claims. Claims 29-32 are directed to a method for preventing and/or treating influenza virus infection or for eliciting long-lasting protective antiviral immune responses against influenza viruses, comprising administering to a patient in need thereof a pharmaceutically effective amount of the composition of claim 20. Based on these claim limitations, the specification only needs to demonstrate that the claimed method would be sufficient to prevent or treat influenza virus infection. As shown in the Examples of the specification, upon administration of the composition of claimed invention, the effects of influenza virus infection was completely prevented.

As shown in the experimental results and discussed on page 10, lines 5-16, of the specification, the efficacy of the naked and liposome-encapsulated pCI-HA10 plasmids to protect animals against lethal challenge of influenza virus by intranasal and intramuscular administrations is shown in FIGS. 3 and 4. Non-immunized mice succumbed to the influenza infection at as early as 7 days post infection, and all animals were dead by day 9. All mice which received intranasal immunization with naked unencapsulated pCI-HA10 also succumbed to the infection, with no increase in survival rate nor survival time (FIG. 3). In contrast, mice **immunized intranasally** with liposome-encapsulated pCI-HA10 *were found to be completely protected with 100% survival rate (p<0.01 vs. control or naked pCI-HA10 group)*. Further, when the pCI-HA10 DNA was **administered by intramuscular injection**, both liposome-encapsulated and naked pCI-HA10 plasmid were shown to provide complete protection against the virus challenge (FIG. 4). In contrast, liposome-encapsulated pCI without the HA insert provided little or no protection (see paragraph 0055 of the publication of the present application).

To provide further experimental data in support of the effectiveness of the present invention, Applicant has submitted additional articles (enclosed herewith) which shows that the claimed invention do provide complete protection in the prevention of influenza virus infection. For instance, Figure 3 in Wong et al. (Vaccine 19:2461-2467) and the article by Wang et al. (Journal of Clinical Virology, 31:S99-S106, 2004), show the vaccine providing at least 1 week protection (Figure 2), and strong T cell responses even at 2 weeks post vaccine administration (see Figure 7).

The Examiner has noted in the Office Action that the enabling disclosure is clearly not commensurate in scope with these claims. However, such a position is unduly restrictive and contrary to that expressed in the Manual of Patent Examining Procedure.

Under § 2164.01(c) of the MPEP, if a statement of utility in the specification contains within it a connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated, 35 USC § 112 is satisfied. *In re Johnson*, 282 F.2d 370, 373, 127 USPQ 216, 219 (CCPA 1960). It is not necessary to specify the dosage or method of use if it is known to one skilled in the art that such information can be obtained without undue experimentation. When a compound or composition claim is limited by a particular use, enablement of that claim should be evaluated based on that limitation. See *In re* Vaeck, 947 F.2d 488, 495, 20 USPQ 2d 1438, 1444 (Fed. Cir. 1991).

Here, in this case, the animal models used in the Examples of the specification clearly support the limitations of the claims. It is well recognized in the art that the particular animal model of the specification correlates with the influenza virus infection in humans. In other words, Applicants submit that the animal model studies of the specification would be viewed by one skilled in the art as being reasonably predictive of the effectiveness of the present invention in treating or preventing influenza virus infection.

It is important to emphasize that the Federal Courts have consistently reversed rejections by the Patent Office asserting a lack of enablement for inventions claiming a pharmacological or therapeutic use where Applicants have provided evidence that reasonably support such a use. As a general rule, evidence of pharmacological or other biological activity of a composition will be relevant to an asserted therapeutic use if there is a ***reasonable correlation*** between the activity of the composition and its asserted method of use. Applicants do not have to prove with statistical

certainty that a correlation exist between a particular activity of a compound or composition and its therapeutic use. Further, Applicants also are not required to provide actual evidence of success in **treating humans** where such utility is asserted. Instead, as the Courts have repeatedly held, all that is required is a reasonable correlation between the activity of the compound or composition, and its asserted use.

Thus, if reasonably correlated to the particular therapeutic or pharmacological activity, data generated using *in vitro* assays or tests in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological use for the compound, composition or process. In other words, while an Applicant may need to provide evidence to show that the invention will work as claimed, it is improper for the Patent Office to question the *"degree of effectiveness"* of a claimed method and/or pharmaceutical composition. See *In re Sichert*, 566 F.2d 1154, 196 USPQ 209 (CCPA 1977); *In re Hartop*, 311 F.2d 249, 135 USPQ 419 (CCPA 1962); *In re Anthony*, 414 F.2d 1383, 162 USPQ 594 (CCPA 1969); *In re Watson*, 517 F.2d 465, 186 USPQ 11 (CCPA 1975); *In re Krimmel*, 292 F.2d 948, 130 USPQ 215 (CCPA 1961); *Ex Parte Jovanovics*, 211 USPQ 907 (Bd. Pat. App. & Inter. 1981).

The Examiner has argued in the Office Action that Sha et al. teach vaccine failure of some plasmid liposome formulations and that the claims do not exclude those embodiments. However, Sha et al. clearly <u>does not teach</u> the plasmid liposome formulations of the present invention. As explained thoroughly in Applicant's previously filed response (see pages 5-8 of the response filed July 13, 2005) which Examiner has agreed with, Sha et al. describes mixing the influenza HA gene with Dosper liposome (see page 22 of Sha et al., *"20μg of pjw4303 DNA was mixed with 40μg of Dosper in HBS"*), and discusses that cationic liposomes have been shown to efficiently <u>complex</u> with DNA (see page 26 of Sha et al.). In other words, Sha et al. clearly describes experiments which were conducted to determine the effectiveness of DNA/lipid <u>complexes</u> and not composition of DNA <u>encapsulated within liposomes</u>. Thus, since Sha et al. clearly does not teach the plasmid liposome formulations of the present invention, the claims clearly exclude the embodiments of Sha et al. Hence, the experimental results of Sha et al. cannot be used to demonstrate the lack of effectiveness of the full scope of the present invention.

As stated above, the disclosure, Examples of the specification, and additional references (Wong et al. and Wang et al.) clearly demonstrate the effectiveness of the claimed method in preventing and/or treating influenza virus infection in humans. Thus, for these reasons, Applicants believe that this rejection of claims 29-32 under 35 USC § 112, first paragraph, cannot be sustained and should be withdrawn.

Rejection under 35 U.S.C. §103

Claims 20-27 and 29-32 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Davis et al. in view of Sha et al. and Promega Catalog. Further, claims 20 and 26-28 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Wheeler et al., Webb et al., Yau-Young, and Davis et al. in view of Sha et al. Applicant respectfully traverses these rejections.

To establish a *prima facie* case of obviousness, the cited references, in combination, must teach or suggest the invention as a whole, including all the limitations of the claims. Here, in this case, the combination of (1) Davis et al. Sha et al. and Promega Catalog, and (2) Wheeler et al., Webb et al., Davis et al. and Sha et al., fails to teach the claimed liposomal vaccine composition (i.e. *a plasmid **encapsulated within a liposome** wherein the liposome is prepared from a lipid film which is mixed with a solution containing said plasmid*) and the method of making thereof.

As previously argued, Applicants submit that there is no motivation in any of the cited references to modify or combine reference teachings. Under U.S. practice, to establish a *prima facie* case of obviousness, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify or combine reference teachings. As noted in Applicant's earlier responses and arguments which the Examiner had found persuasive, a novel and unobvious aspect of the patent invention is that the DNA vaccine is encapsulated within the liposomes, and not complexed with the lipids. Since the application of the Webb et al. reference is for increasing the circulation time and the pharmacokinetics of the anticancer drug, vinersitine (see abstract, page 272, line 6-9, of Webb et al.) in the body and not about encapsulating plasmid DNA vaccine within liposomes, and avoiding DNA-lipocomplex formation, Applicant submits that there is insufficient motivation in the cited references to modify or combine reference teachings.

Further, Davis et al. only contains a general discussion on the use of plasmid DNA vaccines as a background to Davis's CpG oligos technology, and how adjuvants such as CpG oligos could be used to enhance the immune responses to vaccines. The citation in Davis et al. noted by the Examiner (see column 26, lines 15-50, of Davis et al.) only discusses liposomes as a possible delivery system but does not at all disclose encapsulating plasmid DNA vaccine within liposomes. Applicant respectfully request the Examiner to review the teachings of Davis et al. and provide a citation as to where in Davis et al. does it disclose encapsulating plasmid DNA vaccine within liposomes.

It should also be noted that Davis et al. primarily focuses on the use of CpG oligos to enhance the immune responses to hepatitis B vaccine (HBV) which is in conflict with the present invention which does not advocate using CpG oligos or developing an adjuvant to Hepatitis virus vaccine. Hence, it is clear that Davis et al. teaches away from the present invention since one skilled in the art would not be motivated to modify the teachings of Davis et al. to arrive at the present invention. Under U.S. case law, a prior art reference that "teaches away" from the claimed invention is a significant factor to be considered in determining obviousness. In other words, it is improper to combine references where the references teach away from their combination. *In re Grasselli*, 713 F.2d 731, 743, 218 USPQ 769, 779 (Fed. Cir. 1983).

In addition, there is a significant difference between generating an antibody response to a vaccine (Davis et al.) and viral infection protection, because high antibody response does not guarantee protection against virus infections. In other words, it is well known in the art that antigens generating good antibody response are found to be ineffective when tested for efficacy against a real virus challenge in animals and humans. Therefore, modification of the teachings in Davis et al. does not provide a reasonable expectation of success, as required to establish a *prima facie* case of obviousness.

Lastly, in further support of Applicant's position that the prior art references are not in the Applicant's field of endeavor, and are not reasonably pertinent to the particular problem with which the Applicant was concerned, Applicant submits that the endeavour and particular problem being applied in the present invention is vaccine development against infectious diseases while, for example, Webb et al, relates to oncology and cancer therapy. Furthermore, it is also important to

note that the present invention is directed to a DNA vaccine which is unrelated in both biochemical structures and functions to the anticancer drug of Webb et al. Hence, there is clearly no relevance or pertinence to the field of DNA vaccine. In fact, given the differences in encapsulating DNA vaccine versus anticancer drug, Applicant does not believe that the problems and solutions of encapsulating an anticancer drug would relate to the problems associated with encapsulating a DNA vaccine.
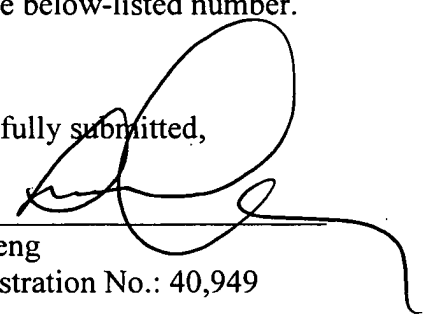
Thus, for these reasons, withdrawal of this rejection is respectfully requested.

CONCLUSION

For the foregoing reasons, all the claims now pending in the present application are believed to be clearly patentable over the outstanding rejections. Accordingly, favorable reconsideration of the claims in light of the above remarks is courteously solicited. If the Examiner has any comments or suggestions that could place this application in even better form, the Examiner is requested to telephone the undersigned attorney at the below-listed number.

Dated: June 22, 2007

Respectfully submitted,

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Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No. 180013 for any such fees; and applicant(s) hereby petition for any needed extension of time.

Intranasal immunization with liposome-encapsulated plasmid DNA encoding influenza virus hemagglutinin elicits mucosal, cellular and humoral immune responses

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Abstract

Background: Influenza viral infections are a significant global public health concern due to the morbidity and mortality associated with acute respiratory disease, associated secondary complications and pandemic threat. Currently, the most effective preventative measure is an annual intramuscular (i.m.) injection of a trivalent vaccine. Intramuscular immunization induces strong systemic humoral responses but not mucosal immune responses which are important in the first line of defense against influenza.
Objectives: A plasmid encoding influenza A/PR/8/34 (H1N1) hemagglutinin (HA; pCI-HA10) was evaluated with respect to the mucosal, cellular and humoral immune responses generated and to its efficacy in protection against a challenge with a lethal dose of influenza.
Study design: BALB/c mice were immunized with pCI-HA10 DNA or liposome-encapsulated pCI-HA10 by either an intranasal (i.n.) or i.m. route. Sera and bronchoalveolar lavage (BAL) fluid were collected at various times and evaluated for HA-specific IgG and IgA antibodies and T cells, isolated from draining lymph nodes and spleens, were analyzed for their proliferative ability. Immunized mice were challenged with a lethal dose ($5LD_{50}$) of influenza and monitored for survival.
Results and conclusions: Intranasal immunization with liposome-encapsulated pCI-HA10 stimulated both IgG and IgA humoral responses and increased IgA titers in BAL fluid, whereas immunization with naked pCI-HA10 had no effect on the antibody response. Intramuscular immunization with both naked and liposome-encapsulated pCI-HA10 elevated serum IgG levels, but had no effect on IgA levels in either the serum or BAL fluid. Both i.n. and i.m. administration of HA vaccine (naked and liosome-encapsulated) elicited T cell proliferative responses. These results suggest that i.n. administration of liposome-encapsulated HA-encoding DNA is effective at eliciting mucosal, cellular and humoral immune responses. Mice immunized i.n. were able to withstand a lethal challenge of influenza virus, confirming that the immune responses mediated by the vaccine were protective.
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Keywords: DNA vaccine; Influenza; Liposomes

1. Introduction

Acute respiratory disease and secondary complications from influenza viral infections are among the leading causes of human morbidity and mortality. Vaccination using inactivated virus remains the most effective preventative measure against influenza (Bridges et al., 2003) however, as the vaccine is grown in embryonated chicken eggs it is not suited for people allergic to egg products. In addition, components of this trivalent vaccine must be adjusted annually as influenza

viruses continually mutate resulting in antigenic changes (Campitelli et al., 2002). Protective immunity provided by the current, parenterally administered influenza vaccine is based on the induction of strain-specific IgG antibodies directed against influenza hemagglutinin (HA), with the vaccine providing optimal protection against viruses that are antigenically closely matched with those of the vaccine (Tumpey et al., 2001). Avian species are the most common natural host of influenza A viruses and passage of avian influenza viruses directly (rarely) or indirectly to humans has the potential to create viruses which are highly transmissible between and virulent to humans, while lacking surface antigens that enable them to be recognized by the immune system (de Jong et al., 1997; Gregory et al., 2001). New emerging virus strains, evolved from recombination, reassortment and/or mutations have potential to initiate a pandemic resulting in massive loss of human lives and devastating effects on the economy.

In animal models, administration of plasmid DNA encoding a variety of viral proteins have been used effectively as DNA vaccines against influenza (Gurunathan et al., 2000, Johnson et al., 2000; Wong et al., 2001), HIV (Jounai et al., 2003), varicella zoster (Stasikova et al., 2003), vaccinia (Hooper et al., 2003), hepatitis B virus (Thermet et al., 2003) and human papillomavirus (Moniz et al., 2003). Enhanced survival, following lethal influenza infection, has been observed following epidermal administration of plasmid mixtures encoding HA, NA and matrix protein M1 (Chen et al., 1999) and i.m. administration of HA (Deck et al., 1997; Johnson et al., 2000; Wong et al., 2001), nucleoprotein (NP; Ulmer et al., 1993), M1 and M2 (Okuda et al., 2001) or i.n. administration of HA (Wong et al., 2001), M1 and M2 (Okuda et al., 2001). As plasmids do not produce infection, the safety concerns inherent with live or attenuated vaccines are eliminated.

The immune response to DNA vaccination appears to mimic that of natural viral infections as plasmid-encoded proteins are produced in their native conformation, processed and presented by antigen presenting cells (APC) in association with MHC class I and class II molecules, to elicit cytotoxic, helper T (Th) cell and humoral immune responses (Gurunathan et al., 2000; Johnson et al., 2000). However, the ability of DNA vaccines to generate cell-mediated and humoral immunity remains controversial. Following i.m. immunization with HA or NP encoding DNA, strong antigen specific IgG (Ulmer et al., 1993; Kadowaki et al., 2000; Wong et al., 2001), IgG and IgA (Deck et al., 1997) or weak antibody responses (Johnson et al., 2000) have been observed.

Since influenza virus primarily infects and multiplies in the upper respiratory tract, elicitation of strong mucosal and cell-mediated immune (CMI) responses are critical in the formulation of an effective influenza vaccine. Mucosal immunization efficiently stimulates mucosal IgA which can form a critical first line of host defense against influenza infection, preventing viral attachment to lung epithelial cells and strengthening the overall immune defense against infectious viral particles and inducing secretion of antigen-specific IgA antibodies in mucosal districts distant from the site of immunization. However, mucosal administration of soluble proteins does not usually induce an immune response unless mucosal adjuvants, such as *Escherichia coli* heat-labile enterotoxin, *Vibrio cholerae* cholera toxin (Wu et al., 1997) or zonula occludens toxin are present (Marinaro et al., 1999). Intranasal immunization stimulates nasal lymphoid tissue (NALT) to seed T and B cells to the draining cervical lymph nodes where large numbers of specific antibody-secreting cells are found along with antigen-specific T cells (Wu et al., 1997). Although a variety of immunization protocols for DNA vaccination have been shown to induce the production of circulating IgG and/or IgA antibodies (Deck et al., 1997; Johnson et al., 2000; Kadowaki et al., 2000; Wong et al., 2001), there is usually little relationship between resistance to mucosa-associated infections and levels of circulating antibodies.

We have previously shown that a DNA vaccine encoding influenza virus HA administered to mice i.m. can provide protection against a lethal dose of influenza without increasing serum IgA levels (Wong et al., 2001). Encapsulation of the DNA within liposomes, together with i.n. administration, provided protection against viral challenge as well as elevating serum IgA levels. The dose required to mediate protection against influenza infection was lower for i.n. than for i.m. immunization, suggesting that mucosal immunity may have a role in the protective effect mediated by i.n. administration (Wong et al., 2001). This observation was of particular interest as influenza virus primarily infects the upper respiratory tract and induction of mucosal immunity appears to have a role in limiting viral attachment to lung epithelial cells.

Considering the ease with which i.n. vaccines can be administered, a study was undertaken to examine the cellular, humoral and mucosal immune response to DNA vaccine. We were interested in ascertaining whether i.n. administration of HA DNA (pCI-HA10) encapsulated within cationic liposomes could activate all three immune responses. We demonstrate that i.n. administration of liposome-encapsulated pCI-HA10 induced T-cell proliferation, indicative of CD4+ activity, in addition to increasing serum IgG and IgA titers, indicative of humoral immune responses. Examination of secretory IgA in bronchoalveolar lavage (BAL) fluid indicated that i.n. administration of liposome-encapsulated DNA was effective at stimulating mucosal immunity. Mice challenged with a lethal dose of influenza virus following i.n. immunization with liposome-encapsulated pCI-HA10 were completely protected. Together these results suggest that delivery of a liposome-encapsulated DNA vaccine to the respiratory tract can induce protective immunity against viral respiratory infections.

2. Materials and methods

2.1. Liposome encapsulation of DNA

Plasmid DNA encoding influenza A virus HA (pCI-HA10) was encapsulated in cationic liposomes and particle size

and zeta potential were determined as previously described (Wong et al., 2001). The integrity of the encapsulated DNA was evaluated by agarose gel electrophoresis, and the efficacy of encapsulation was confirmed by protection from DNAse 1 (Invitrogen, Burlington, Ont.) digestion.

2.2. Gene expression in vitro

African green monkey kidney epithelial (COS-1; ATCC, Manassas, VA) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen). For transfection studies, COS-1 cells were grown overnight at 37 °C with 5% CO_2 in 12-well culture dishes. Wells containing adherent cells at 60–70% confluency were washed twice with DMEM and transfected in the presence of 10 μL Superfect (Qiagen, Mississauga, Ont.) complexed with 2 μg of pCI-HA10. HA expression was determined 48 h after transfection. Transfected COS-1 cells were treated with a blocking solution [20% (v/v) FBS, 3% (w/v) dry milk and 0.5% (v/v) Tween-20 in phosphate buffered saline (PBS)], incubated with a 1:200 dilution of anti-influenza type A HA IgG monoclonal antibody (Biodesign Intl., Kennebunk, ME), washed to remove unbound antibody and incubated with peroxidase-conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD). Peroxidase activity was detected using liquid 3,3′-diaminobenzidine chromogen solution (BioGenex, San Ramon, CA) and the number of peroxidase positive cells was determined microscopically. Protein content of cell lysates and tissue samples were determined using a Bio-Rad protein assay (Bio-Rad, Mississaga, Ont.).

2.3. DNA vaccination of mice

Eight- to twelve-week-old female BALB/c mice were obtained from the mouse-breeding colony at Defence Research and Development Canada—Suffield (DRDC Suffield). The use of these animals was reviewed and approved by the DRDC Suffield Animal Care Committee and the care and handling of these animals followed guidelines set out by the Canadian Council of Animal Care.

Mice were immunized with naked or liposome-encapsulated pCI-HA10 using intramuscular (i.m.) or intranasal (i.n.) routes of administration. For i.m. immunization, either 40 μg liposome-encapsulated DNA or 150 μg naked DNA mice was injected into the quadriceps muscle. For i.n. immunization, mice were anesthetized with sodium pentobarbital (50 mg/kg body weight) by intraperitoneal (i.p.) injection. Twenty microgram (50 μL) of the DNA preparation was administered gently into one of the nostrils with a micropipettor when the animals were completely unconscious. The applied volume was naturally inhaled into the lungs. Any mice observed swallowing (an indicator that the substance administered had not been inhaled into the lungs) were removed from the experiment. Intranasal immunization was repeated on day 3 for a total dose of 40 μg DNA

administered. Both i.m. and i.n. groups received an additional boost on day 14 (i.m.) and days 14 and 16 (i.n.). Mice were euthanized on day 28 and serum, lungs, draining lymph nodes, spleen and BAL fluid were collected for immunological analysis. Lungs were rinsed three times with PBS, and then 400 μL PBS was injected through the trachea. BAL fluid was collected by squeezing the lungs into a dish. Approximately 100–150 μL of BAL fluid was obtained from each mouse.

For vaccine efficacy studies, mice were immunized with 50 μL containing 20 μg or 50 μg of the DNA preparation i.n. or i.m., respectively, with three additional boosts of DNA vaccine given 4 weeks apart. Control mice were administered PBS. Blood was collected 1 week after each boost, allowed to clot at room temperature, centrifuged, and the sera was removed and stored at −20 °C.

2.4. Virus challenge of immunized mice

For virus challenge studies, an egg-propagated, mouse-adapted strain of influenza A/PR/8/34 (H1N1) (Wong et al., 2001) was used. Mice immunized with naked or liposome-encapsulated pCI-HA10 were challenged i.n., 1 week following the last booster dose, with 5 LD_{50} of virus. The mice were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) and when completely unconscious 50 μL of the egg-propagated virus was administered gently into one nostril. At 14 days post-infection, the numbers of surviving mice in each of the control and test groups were recorded. Ten mice were used in each test group.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was used for evaluation of antibody response essentially as previously described (Wong et al., 2001). Microtitre plates (96 wells, Costar, Cambridge, MA) were coated with influenza virus A/PR/8/34 (500 ng in 50 μL 50 mM carbonate buffer [pH 9.6] per well) at 4 °C for 18 h. Wells were blocked with 2% (w/v) bovine serum albumin (BSA), 1% (v/v) Tween-20 in PBS for 1 h at 37 °C, incubated with serial dilutions of mouse serum for 1 h at 37 °C, followed by incubation with peroxidase-labeled goat anti-mouse IgG and anti-mouse IgA (Kirkegaard & Perry, Gaithersburg, MD) for 1 h at 37 °C. The plates were thoroughly washed between each step with multiple washes of 0.1% (w/v) BSA, 1% (v/v) Tween-20 in PBS. Peroxidase activity was measured using 2,2′-azino-di[3-ethyl-benzthiazoline sulfonate] (Kirkegaard & Perry) as a substrate and determined at 405 nm (Bio-Tek Inc., Winoosky, VT) after 30 and 60 min incubation at room temperature in the dark. Results were analyzed with SoftMax Pro.

2.6. Measurement of T cell responses

T cells were isolated from draining lymph nodes and spleens of immunized mice by passage through nylon wool

(type 200 L, Robbins Scientific, Sunnyvale, CA) inserted into a Luer-lok syringe (BD Luer-lok Plastipak, Rutherford, NJ). T cells were eluted from the column with supplemented RPMI 1640 medium [RPMI 1640 medium (Invitrogen) supplemented with 1 mM L-glutamine (Invitrogen), 55 µM 2-mercaptoethanol (Invitrogen), 50 mU/mL penicillin G, sodium salt (Invitrogen), 50 ng/mL streptomycin sulfate (Invitrogen), 75 µg/mL gentamicin (Invitrogen), 10 mM HEPES (Invitrogen) and 10% (v/v) FBS], centrifuged at 200 × g for 8 min and resuspended at 1 × 10^7 cells/mL in supplemented RPMI 1640 medium. APCs were obtained from the spleens of unimmunized syngeneic mice. Spleen cells collected in supplemented RPMI 1640 medium were irradiated with ^{137}Cs (3000 rad). Following centrifugation at 200 × g for 8 min, irradiated spleen cells were resuspended in supplemented RPMI 1640 medium at a concentration of 1 × 10^7 cells per well.

For T cell proliferation assays, T cells were plated (1 × 10^6, 5 × 10^5 and 2 × 10^5 T-cells per well) in 96-well microtiter plates (Costar). Test antigens (ovalbumin, A/PR/8/34 HA; 25 ng to 2.5 µg) and APCs (1 × 10^6 cells) were added to each well. Positive controls (2 × 10^5 T cells per well) were incubated with 2 µg of the mitogen concanavalin A (Con A, Sigma). The plates were incubated at 37 °C and 5% CO_2 for 96 h, pulsed with [^{3}H]-thymidine (1 µCi per well, Amersham, Oakville, Ont.) for 24 h and harvested onto 96-well-format filter mats. Incorporated [^{3}H]-thymidine was measured in a Microbeta counter (Wallac, Finland). T cell proliferation (stimulation index) was determined by comparison of CPM in wells containing recall antigen with CPM in wells lacking recall antigen. All assays performed in triplicate and the experiment was replicated three times.

2.7. Statistics

Serum IgG and IgA levels and survival rates following viral challenge in the immunized mice were compared using the Mann–Whitney unpaired, two-tailed test (GraphPad Prism v2.01, GraphPad Software Inc., San Diego, CA). Results were considered to be significant when $p \leq 0.05$.

3. Results

3.1. Characterization of encapsulated DNA

Liposomes averaged 98.3 ± 6.1 nm in size and contained 0.73 ± 0.23 mg DNA per mL of liposomes, for a mean encapsulation efficiency of 48.8 ± 8.3%. The liposome-encapsulated DNA retained structural and functional integrity as determined by gel electrophoresis and transfection of COS-1 cells and liposome encapsulation was able to protect plasmid DNA from DNAse I digestion (data not shown).

3.2. Immune responses to DNA vaccine

Previous studies have suggested that i.n. immunization with a DNA vaccine encoding influenza virus HA may evoke a mucosal immune response which is absent when the vaccine is administered i.m. (Wong et al., 2001). Preliminary studies performed following vaccination with a reporter gene suggested that a DNA vaccine administered i.n. could induce CMI and that encapsulation within liposomes enhanced the immune response (unpublished data). As our primary goal was to formulate an efficacious DNA vaccine against influenza we wanted to analyze in detail the immune and survival responses generated by vaccination with a plasmid encoding influenza HA (pCI-HA10).

The efficacy of i.m. and i.n. administration of naked and liposome-encapsulated pCI-HA10 to protect mice against a lethal challenge with influenza A/PR/8/34 virus is shown in Figs. 1 and 2, respectively. Non-immunized mice succumbed to influenza as early as 7 days post-infection and all mice were dead by day 10 (Figs. 1A and 2A). Increasing the number of boosts, administered 4 weeks apart, in mice immunized i.m. resulted in a progressive increase in survival rate (data not shown) with one primary and three boosting doses providing 100% protection for mice administered both naked and
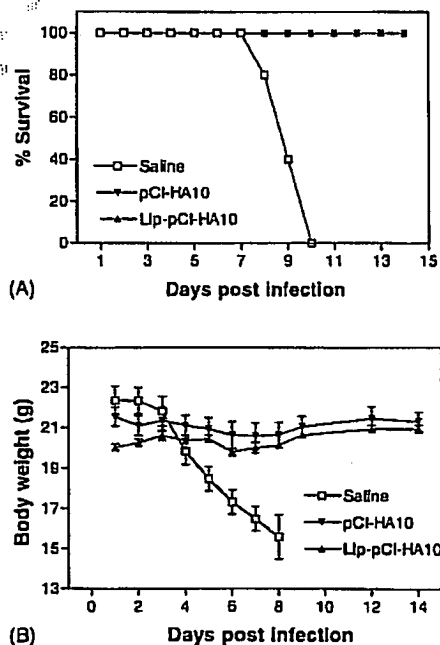

(A) Days post infection

(B) Days post Infection

Fig. 1. Efficacy of i.m. administration of naked or liposome-encapsulated pCI-HA10 in protecting mice against infection with 5 LD$_{50}$ of mouse-adapted influenza A/PR/8/34. Mice were immunized i.m. with one primary and three booster doses of naked pCI-HA10 or liposome-encapsulated pCI-HA10 (Lip-pCI-HA10), 50 µg DNA per dose. Control mice were administered PBS. One week after the final immunization boost, the mice were challenged i.n. with 5 LD$_{50}$ of influenza virus. Survival and body weights were monitored daily for 14 days. (A) Percentage survival, (B) average body weight ± S.D. The experiment was repeated two times.
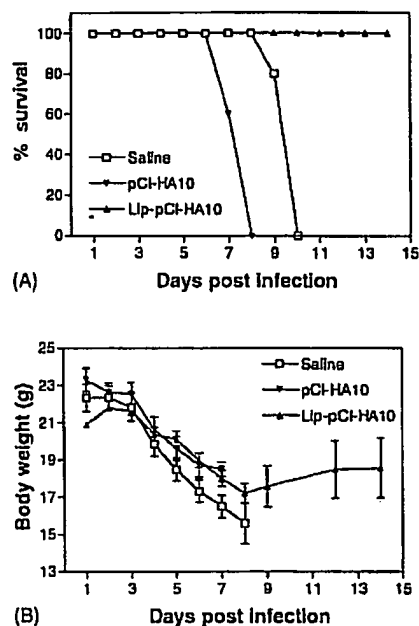
(A) Days post Infection

(B) Days post Infection

Fig. 2. Efficacy of i.n. administration of naked or liposome-encapsulated pCI-HA10 in protecting mice against infection with 5 LD_{50} of mouse-adapted influenza A/PR/8/34. Mice were immunized i.n. with one primary and three booster doses of naked pCI-HA10 or liposome-encapsulated pCI-HA10 (Lip-pCI-HA10), 20 μg DNA per dose. Control mice were administered PBS. One week after the final immunization boost, the mice were challenged i.n. with 5 LD_{50} of influenza virus. Survival and body weights were monitored daily for 14 days. (A) Percentage survival, (B) average body weight ± S.D. The experiment was repeated two times.

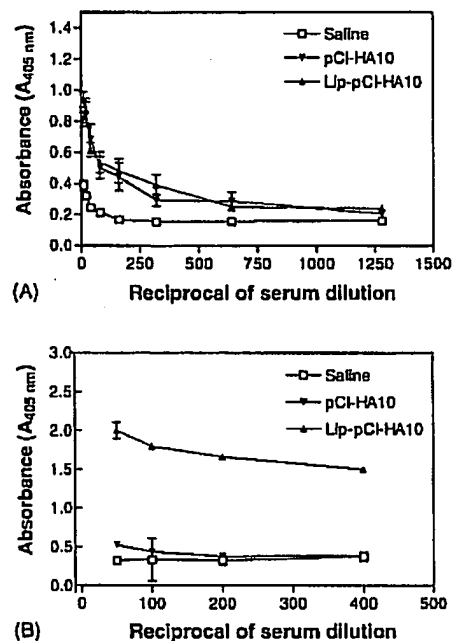**(A)** Reciprocal of serum dilution

(B) Reciprocal of serum dilution

Fig. 3. Serum IgG titers of mice immunized (A) i.m. or (B) i.n. with naked and liposome-encapsulated pCI-HA10. Each mouse received one primary and three booster doses (50 μg/dose i.m., 20 μg/dose i.n.), 4 weeks apart, of naked pCI-HA10 or liposome encapsulated (Lip-pCI-HA10). Control mice administered PBS. Mice were tail bled 1 week after the final immunization and HA-specific IgG titers in the serum were determined by ELISA. Each point represents the mean ± S.D. The experiment was replicated two times.

liposome-encapsulated pCI-HA10 (Fig. 1A). Body weights of these two groups of mice did not significantly change following infection with a lethal dose of influenza (Fig. 1B). Mice immunized i.n. with liposome-encapsulated pCI-HA10 had a survival rate of 100% 14 days post-infection whereas naked pCI-HA10, administered i.n., was ineffective at increasing survival (Fig. 2A). Body weights of mice immunized i.n. with liposome-encapsulated pCI-HA10 progressively decreased during the first 8 days post-infection, followed by a gradual recovery (Fig. 2B).

HA-specific IgA and IgG titers in serum samples of mice were determined by indirect ELISA. Mice immunized i.m. with both naked and liposome-encapsulated pCI-HA10 had a significant increase in serum IgG titer (Fig. 3A). Mice immunized i.n. with liposome-encapsulated pCI-HA10 had a significant increase in serum IgG titer, whereas immunization with naked pCI-HA10 had no effect (Fig. 3B). IgG subtyping of HA-specific IgG antibodies following i.m. administration indicated that IgG1, IgG2a, IgG2b and IgG3 titers were all increased (Fig. 4). The IgG2a:IgG1 ratio was 2.1 ± 0.15 indicating a Th1 type response. Mice immunized with liposome-encapsulated pCI-HA10 by the i.n. route were found to have a significant increase in serum IgA titers (Fig. 5). Increases in HA-specific IgA antibody were not detected in sera from mice immunized i.n. with naked pCI-HA10. Intramuscular immu-

nization of mice with either naked or liposome-encapsulated pCI-HA10 did not result in an increase of serum IgA (data not shown).

Secretory IgA was induced in mice immunized i.n. with naked and liposome-encapsulated pCI-HA10 (Fig. 6). This observation is significant as it indicates that i.n. administration of vaccine is effective at inducing mucosal immunity.

To study whether i.n. administration of vaccine stimulated an enhanced CMI response, T cell proliferation was measured. Mice were treated with naked and liposome-encapsulated pCI-HA10. Fourteen days after the second im-
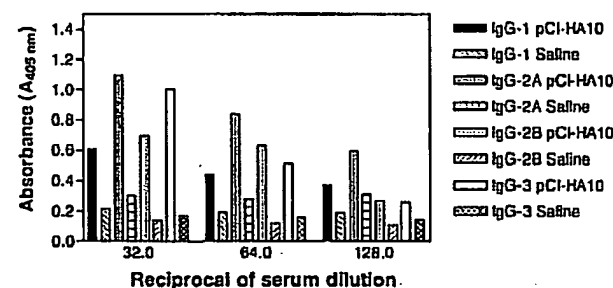

Reciprocal of serum dilution

Fig. 4. Serum IgG subtype titers of mice immunized i.m. with naked and liposome-encapsulated pCI-HA10. Mice were immunized and sera was collected as described in Fig. 3. HA-specific IgG1, IgG2a, IgG2b and IgG3 titers in the serum samples were determined by ELISA.
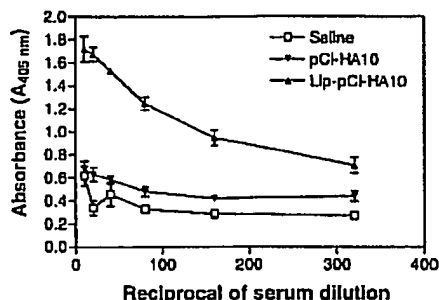
Fig. 5. Serum IgA levels of mice immunized i.n. with naked and liposome-encapsulated pCl-HA10. Mice were immunized and sera was collected as described in Fig. 3. HA-specific IgA titers in the serum samples were determined by ELISA. Each point represents the mean ± S.D. The experiment was replicated two times.

munization, spleen cells were collected and co-cultured with A/PR/8/34 HA and syngeneic spleen cells. Strong T cell proliferative responses were detectable in mice immunized with naked or liposomal pCl-HA10 by both the i.m. and i.n. routes (Fig. 7). No significant proliferation was noted in the presence of irrelevant recall antigens (data not shown). Although i.m. immunization with pCl-HA10 had a stronger T cell proliferative response than i.n. immunization, these results demonstrate that a significant CMI response is induced with i.n. immunization.

In summary, mice immunized with the liposome-encapsulated formulation of pCl-HA10 by the i.n. route had 100% survival rate following challenge with a lethal dose of influenza virus (Fig. 2), a demonstrable increase in specific IgA antibodies in both the serum (Fig. 5) and BAL fluid (Fig. 6), and a significant increase in specific IgG antibodies in their sera (Fig. 3). Additionally, a specific T cell proliferative response, indicative of T helper cell activation, was observed (Fig. 7), suggesting that the liposome-encapsulated formulation of pCl-HA10 evaluated in this study was able to induce CMI.

The above studies show that liposome-encapsulated pCl-HA10 is an effective DNA vaccine delivery system for the in-
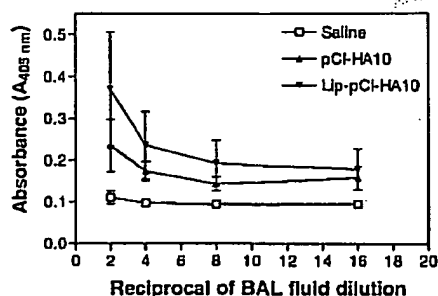

Fig. 6. IgA levels of BAL fluid from mice immunized i.n. with naked and liposome-encapsulated pCl-HA10. Mice were immunized as described in Fig. 3. BAL fluid was collected 1 week after the final immunization. HA-specific IgA titers were determined by ELISA. Each point represents the mean ± S.D.
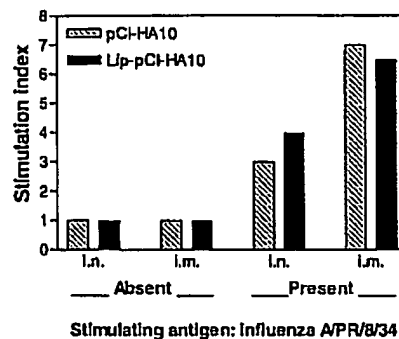

Stimulating antigen: Influenza A/PR/8/34

Fig. 7. T cell proliferation activity in mice immunized i.m. or i.n. with naked and liposome-encapsulated pCl-HA10. Mice were immunized with 150 μg of pCl-HA10 i.m. or 40 μg liposome-encapsulated pCl-HA10 i.m., 40 μg pCl-HA10 i.n. or 40 μg liposome-encapsulated pCl-HA10 i.n. twice, 2 weeks apart. Two weeks after the last immunization, lymphocytes were collected from the spleen and draining lymph nodes. Isolated T cells (1 × 10^6 cells per well) were incubated with 25 ng to 2.5 μg of A/PR/8/34 HA and 1 × 10^7 irradiated syngeneic spleen cells. T cell proliferation was assayed by [^3H]thymidine incorporation into proliferating cells. Results are reported as stimulation index (CPM incorporated in wells containing recall antigen/CPM incorporated into wells without recall antigen). All assays were performed in triplicate. The experiment was repeated three times.

duction of antigen-specific mucosal immune responses. The responses induced by immunization with plasmid DNA encoding HA included both T cell mediated and humoral responses that were specific to influenza virus A/PR/8/34.

4. Discussion

The development of an effective vaccine that generates long-term protective immunity, can be administered without needle injection and can be mass produced without the use of egg products is an important goal for influenza vaccine development. In this study, we evaluated the humoral, mucosal and cell mediated immune responses, in mice, to influenza HA after vaccination with a liposome-encapsulated plasmid containing a gene encoding HA. The i.n. route of administration was utilized to maximize the possibility of inducing a mucosal immune response. Liposome-encapsulation of HA DNA was utilized to protect the DNA from nuclease degradation in vitro (data not shown) and in the lungs (Mahato et al., 1998) therefore increasing body retention times and enhancing immunological responses. Liposomes also facilitate uptake by antigen-presenting cells, thereby increasing transfection efficiency.

In this study, i.m. administration of naked or liposome-encapsulated HA DNA was completely protective against a lethal challenge dose of influenza, increased serum IgG but had no effect on serum or secretory IgA titers (data not shown). IgG subtyping showed an increase in IgG1, IgG2a, IgG2b and IgG3 with an increase in the ratio of IgG2a:IgG1 from 1.42 ± 0.03 in mice immunized with PBS to 2.1 ± 0.15 in mice immunized with HA DNA, either naked or liposome-

encapsulated, indicating a Th1 type response. A Th1 type response has also been reported following i.m. immunization with a plasmid encoding mycobacterial antigens (Quinn et al., 2002) or influenza virus (Johnson et al., 2000). The strain of mice used in immunization studies may affect the Th response observed as it has been reported that BALB/c mice tend to generate a Th1 response and C57BL/6 and CBA/Ca mice tend to generate Th1 and Th2 responses (Graham et al., 1998).

Examination of HA-specific secretory IgA titers in BAL fluid showed increased IgA titers following i.n. administration of liposome-encapsulated or naked HA DNA, indicating a mucosal humoral response. In a survery of patients undergoing tonsillectomy, large numbers of influenza-specific antibody secreting cells were present in nasal mucosa even when influenza had not recently circulated suggesting that these cells were important in protecting against reinfection with influenza (Brokstad et al., 2002). Inactivated vaccine does not stimulate a mucosal response, although some rise in local antibodies may be seen because of crossover from the systemic response. IgA, the dominant antibody in the upper respiratory tract is actively transported into the respiratory tract from the adenoid and B cells living in crypts in the adenoids and tonsils (Wright, 2002). It has been postulated that in a common mucosal immune system stimulation of one compartment (mucosa) can lead to protection in another (serum or other mucosal tissue). IgG is the dominant antibody in the lung, originating in the serum and leaking across a gradient to the mucosal surface. It has been postulated that inflammation and destruction of epithelial borders during influenza increases the amount of IgG that leaks into the respiratory tract (Wright, 2002). Intranasal administration of an HIV DNA vaccine elicited high mucosal IgA antibody titers in the intestine and vagina (Okada et al., 1997) and i.n. immunization with recombinant influenza virus lead to an increase in IFN-γ secreting cells in the urogenital tract (Ferko et al., 2001). This i.n. stimulation of distal mucosal tissues is speculated to be mediated by nasal-associated lymphoid tissue (Ferko et al., 2001).

Cellular immunity is induced in response to i.n. and i.m. administration of HA DNA vaccine. T cell proliferation was significantly enhanced by DNA vaccination with i.m. administration generating a very high proliferative response. Intranasal administration increased T cell proliferative activity with liposome-encapsulation mitigating a slightly stronger response. T cell proliferation and cytotoxic T lymphocytes are indicators of CD4+ and CD8+ T cells, respectively. CD4+ cells recognize viral peptides presented by MHC class II molecules and, after activation, they function to promote B cell survival and antibody production. Activated CD4+ cells also provide helper function to CD8+ cells and secrete cytokines, which modify the antibody subclass of the humoral response. CD8+ cytotoxic T-lymphocyte activation results in killing of virally infected cells following recognition and presentation by MHC class I molecules. Thus i.n. administration of liposome-encapsulated vaccine results in synthesis of viral

proteins in their native state, such that they can be recognized and processed by both MHC class I and class II molecules to induce both cellular and humoral immunity.

B cells are required for heterosubtypic protection against a lethal influenza virus infection as mice immunized with a formalin-fixed H3N2 strain and treated with monoclonal antibodies to deplete CD4+ and CD8+ T cell populations prior to infection with an H5N1 strain were able to survive infection whereas B cell deficient mice succumbed to infection (Tumpey et al., 2001). This may relate to the functional role of B cells (secretion of cytokines, antigen presentation) and/or the production of cross-reactive antibodies. CD8+ cytotoxic T cells have been implicated in heterosubtypic immunity possibly mediating their effect indirectly by the secretion of antiviral cytokines such as IFN-γ and TNF-α (O'Neill et al., 2000; Tumpey et al., 2001). Conversion of the CD4+ Th response from Th2 to Th1 may also be involved (Moran et al., 1999). In this study, although hetersubtypic immunity was not directly challenged, immunization with liposome-encapsulated HA DNA did demonstrate a shift toward a Th1 response and involvement of B memory cells.

In conclusion, i.n. immunization with liposome-encapsulated HA encoding DNA appears to be an effective means to produce protective immunity in mice by inducing cellular, humoral and mucosal immune responses.

References

Bridges CB, Harper SA, Fukuda SA, Uyeki TM, Cox NJ, Singleton JA. Prevention and control of influenza: recommendations of the advisory committee on immunization practices (ACIP). Morbid Mortal Wkly Rep 2003;52(RR08):1–36.

Brokstad KA, Eriksson J-C, Cox RJ, Tynning T, Olofsson J, Jonsson R, Davidsson Å. Parenteral vaccination against influenza does not induce a local antigen-specific immune response in the nasal mucosa. J Infect Dis 2002;185:878–84.

Campitelli L, Fabiani C, Puzelli S, Fioretti A, Foni E, De Marco A, Krauss S, Webster RG, Donatelli I. H3N2 influenza viruses from domestic chicken in Italy: an increasing role for chickens in the ecology of influenza? J Gen Virol 2002;83:413–20.

Chen Z, Matsuo K, Asanuma H, Takahashi H, Iwasaki T, Suzuki Y, Aizawa C, Kurata T, Tamura S. Enhanced protection against a lethal influenza virus challenge by immunization with both hemagglutinin- and neuraminidase-expressing DNAs. Vaccine 1999;17:653–9.

de Jong JC, Claas ECJ, Osterhaus ADME, Webster RG, Lim WL. A pandemic warning? Nature 1997;389:554.

Deck RR, DeWitt CM, Donnelly JJ, Liu MA, Ulmer JB. Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. Vaccine 1997;15:71–8.

Ferko B, Stasakova J, Sereinig S, Romanova J, Katinger D, Niebler B, Katinger H, Egorov A. Hyperattenuated recombinant influenza A virus nonstructural-protein-encoding vectors induce human immunodeficiency virus type 1 Nef-specific systemic and mucosal immune responses in mice. J Virol 2001;75:8899–908.

Graham CM, Smith CA, Thomas DB. Novel diversity in Th1, Th2 type differentiation of hemagglutinin-specific T cell clones elicited by natural influenza virus infections in three major haplotypes (H-2^{b,d,k}). J Immunol 1998;161:1094–103.

Gregory V, Lim W, Cameron K, Bennett M, Marozin S, Klimov A, Hall H, Cox N, Hay A, Lin YP. Infection of a child in Hong Kong by

527 an influenza A H3N2 virus closely related to viruses circulating in
528 European pigs. J Gen Virol 2001;82:1397–406.

529 Gurunathan S, Klinman DM, Seder RA. DNA vaccines: Immunology,
530 application, and optimization. Annu Rev Immunol 2000;18:927–
531 74.

532 Hooper JW, Custer DM, Thompson E. Four-gene-combination DNA vac-
533 cine protects mice against a lethal vaccinia virus challenge and elic-
534 its appropriate antibody responses in nonhuman primates. Virology
535 2003;306:181–95.

536 Johnson PA, Conway MA, Daly J, Nicolson C, Robertson J, Mills
537 KHG. Plasmid DNA encoding influenza virus haemagglutinin in-
538 duces Th1 cells and protection against respiratory infection despite
539 its limited ability to generate antibody responses. J Gen Immunol
540 2000;81:1737–45.

541 Jounai N, Okuda K, Kojima Y, Toda Y, Hamajima K, Ohba K, Klinman
542 D, Xin KQ. Contribution of the rev gene to the immunogenicity of
543 DNA vaccines targeting the envelope glycoprotein of HIV. J Gene
544 Med 2003;5:609–17.

545 Kadowaki S, Chen Z, Asanuma H, Aizawa C, Kurata T, Tamura S. Pro-
546 tection against influenza virus infection in mice immunized by ad-
547 ministration of hemagglutinin-expressing DNAs with electroporation.
548 Vaccine 2000;18:2779–88.

549 Mahato RI, Anwer K, Tagliaferri F, Meaney C, Leonard P, Wadhwa MS,
550 Logan M, French M, Rolland A. Biodistribution and gene expression
551 of lipid/plasmid complexes after systemic administration. Hum Gene
552 Ther 1998;9.

553 Marinaro M, Di Tommaso A, Uzzau S, Fasano A, De Magistris MT.
554 Zonula occludens toxin is a powerful mucosal adjuvant for intranasally
555 delivered antigens. Infect Immunol 1999;67:1287–91.

556 Moniz M, Ling M, Hung CF, Wu TC. HPV DNA vaccines. Front Biosci
557 2003;8:D55–68.

558 Moran TM, Park H, Fernandez-Sesma A, Schulman JL. Th2 responses
559 to inactivated influenza virus can be converted to Th1 responses and
560 facilitate recovery from heterosubtypic virus infection. J Infect Dis
561 1999;180:579–85.

562 O'Neill E, Krauss SL, Riberdy JM, Webster RG, Woodland DL. Het-
563 erologous protection against lethal A/HongKong/156/97 (H5N1) in-
564 fluenza virus infection in C57BL/6 mice. J Gen Virol 2000;81:2689–
96.

565 Okuda E, Sasaki S, Ishii N, Aoki I, Yasuda T, Nishioka K, Fukushima J,
566 Miyazaki J, Wahren B, Okuda K. Intranasal immunization of a DNA
567 vaccine with IL-12- and granulocyte-macrophage colony-stimulating
568 factor (GM-CSF)-expressing plasmids in liposomes induces strong
569 mucosal and cell-mediated immune responses against HIV-1 antigens.
570 J Immunol 1997;159:3638–47.

571 Okuda K, Ihata A, Watabe S, Okada E, Yamakawa T, Hamajima K,
572 Yang J, Ishii N, Nakazawa M, Okuda K, Ohnari K, Nakajima K, Xin
573 K-Q. Protective immunity against influenza A virus induced by im-
574 munization with DNA plasmid containing influenza M gene. Vaccine
575 2001;19:3684–91.

576 Quinn A, Jiang W, Velaz-Faircloth M, Cobb AJ, Henry SC, Frothingham
577 R. In vivo protein expression and immune responses generated by
578 DNA vaccines expressing mycobacterial antigens fused with a reporter
579 protein. Vaccine 2002;20:3187–92.

580 Stasikova J, Kutinova L, Smahel M, Nemeckova S. Immunization with
581 Varicella-zoster virus glycoprotein E expressing vectors: comparison
582 of antibody response to DNA vaccine and recombinant vaccinia virus.
583 Acta Virol 2003;47:1–10.

584 Thermet A, Rollier C, Zoulim F, Trepo C, Cova L. Progress in
585 DNA vaccine for prophylaxis and therapy of hepatitis B. Vaccine
586 2003;21:659–62.

587 Tumpey TM, Renshaw M, Clements JD, Katz JM. Mucosal delivery of
588 inactivated influenza vaccine induces B-cell dependent heterosubtypic
589 cross-protection against lethal influenza A H5N1 virus infection. J
590 Virol 2001;75:5141–50.

591 Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ,
592 Gromkowski SH, Deck RR, DeWitt CM, Friedman A. Heterologous
593 protection against influenza by injection of DNA encoding a viral
594 protein. Science 1993;259:1745–9.

595 Wong JP, Zabielski MA, Schmaltz FL, Brownlee GG, Bussey LA, Mar-
596 shall K, Borralho T, Nagata LP. DNA vaccination against respiratory
597 influenza virus infection. Vaccine 2001;19:2461–7.

598 Wright PF. Mucosal immunity and the role of the IgA antibody. Inside
599 Influenza 2002;2:1–4.

600 Wu H-Y, Nikolova EB, Beagley KW, Eldridge JH, Russell MW. De-
601 velopment of antibody-secreting cells and antigen-specific T cells in
602 cervical lymph nodes after intranasal immunization. Infect Immunol
603 1997;65:227–35.

DNA vaccination against respiratory influenza virus infection

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Abstract

DNA vaccination using plasmid encoding the hemagglutinin (HA) gene of influenza A/PR/8/34 virus to induce long-lasting protective immunity against respiratory infection was evaluated in this study. Using liposomes as carriers, the efficacy of DNA vaccines was determined using a lethal influenza infection model in mice. Mice immunized intranasally or intramuscularly with liposome-encapsulated pCI plasmid encoding HA (pCI-HA10) were completely protected against an intranasal 5 LD_{50} influenza virus challenge. Mice immunized with liposome-encapsulated pCI-HA10, but not naked pCI-HA10, by intranasal administration were found to produce high titers of serum IgA. These results suggest DNA vaccines encapsulated in liposomes are efficacious in inducing complete protective immunity against respiratory influenza virus infection. Crown Copyright © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: DNA vaccine; Influenza; Liposomes; Immunity

1. Introduction

Despite recent advances in antiviral chemotherapy and vaccine development, infection and complications from influenza remain a leading cause of human mortality or morbidity world-wide. For the elderly and individuals with underlying medical problems, including immunocompromised conditions, influenza can be highly fatal. Vaccination using killed whole virus remains the most effective preventive measure against influenza [1]. However, a major problem with the current influenza vaccines is that they may be ineffective against new variants of the viruses resulting from genetic changes such as antigenic drifts in the hemagglutinin (HA) protein or antigenic shifts to another HA subtype.

Genetic vaccination using plasmid DNA represents an exciting means of inducing protective immunity against viral infections. This new generation of vaccines provides many advantages over conventional live or killed vaccines. Unlike live or attenuated vaccines, DNA vaccines do not produce infection and therefore do not pose inherent safety concerns associated with live or attenuated vaccines. In addition, unlike most subunit vaccines which induce either humoral or cell-mediated immunity, DNA vaccines can stimulate both humoral and cellular immune responses [2,3]. These attributes make DNA vaccines attractive and promising candidates for respiratory viruses, including influenza.

Efficient expression of genes in the plasmid DNA encoding the protective antigens requires the physical uptake of the plasmid by the target cells. Although there is ample evidence which suggests that naked plasmid DNA injected directly into the muscles can express the gene of interest and induce protective immunity [2–6], its ability to be taken up by mucosa-associated lymphoid tissues (MALT) and to induce mucosal immunity has not been well documented. Furthermore, needle injection of the DNA vaccines in muscles in humans can be painful and may present health-related safety issues.

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Since influenza viruses primarily infect and multiply in the lower respiratory tract, delivery of DNA vaccines to the respiratory tract may result in the induction of a focused long-lasting protective immunity in the lungs. Furthermore, the lymphoid tissues found in the large mucosal surfaces in the respiratory tract may enable mucosal immunity to be induced, thereby may help to prevent the virus attachment of the lung epithelial cells and strengthen the overall immune defense against the infectious virus particles.

The objective of this study is to evaluate the pulmonary delivery of liposome-encapsulated DNA vaccine for its ability to confer protection against respiratory virus challenge in mice, and to determine whether such delivery can induce strong and specific mucosal immunity in the respiratory tract. Such an approach may result in the possibility of developing a DNA vaccine formulated in liposomes which could be delivered to the respiratory tract by aerosol inhalation, and may provide strong protective immunity against respiratory influenza viruses.

2. Materials and methods

2.1. Cloning and characterization of hemagglutinin (HA) gene

The original HA construct from influenza A/PR/8/34, P8H has been previously described [7]. The HA was excised with HindIII and BamHI followed by subcloning into pT7-6 [8] to give pT76-HA16, which expresses the HA gene from a T7 promoter. The HA gene was re-amplified from the template pT76-HA16 clone using the following primers: HAXba5', sense (5' TATC-TAGACAAAAGCAGGGGAAAATAAAACAACCA-AAATG 3'); HANot3', antisense, (5'AAGTCAT-AGCGGCCGCAAGGGTGTTTTTCCTCATATTTCT 3'). The *Xba* I and *Not* I sites in HAXba5' and HANot3', respectively, are in italics. Amplification of the HA gene was accomplished by polymerase chain reaction (PCR) using the GeneAmp XL PCR kit with rTth DNA polymerase (Perkin Elmer, Foster City, CA) followed by column purification using the QIAquick PCR purification kit (Qiagen, Valencia, CA). The amplified HA gene was further digested with *Xba* I and *Not* I to create the respective sites at the 5' and 3' ends. The HA gene was then ligated into the pCI vector (Promega Corporation, Madison, WI) at *Xba* I and *Not* I restriction sites, and transformed into competent *E. coli* DH5α cells (Gibco BRL, Bethesda, MD). The pCI-HA10 clone was identified as containing the full-length HA gene, by restriction mapping and DNA sequencing (data not shown). In vitro transcription and translation of the pCI-HA10 clone was performed using the TNT coupled system (Promega) and canine microsomes (Promega) as described by Long et al. [9].

Bulk preparations of pCI-HA10 were prepared with the Endofree Plasmid Mega and Giga kits (Qiagen) following manufacturer's directions and analyzed by restriction enzyme digests. Only plasmid DNA of greater than 90% purity was used for liposome encapsulation and animal immunizations.

2.2. Liposome encapsulation of pCI-HA10

Cationic liposomes used for the encapsulation of pCI-HA10 were prepared using a modification of a procedure previously described [10]. Briefly, liposomes consisting of 7% 1,2-dioleoyl-3-dimethylammonium chloride (DODAC, Avanti Polar Lipid Inc., Alabaster, Alta), 78% 1,2-dioleoyl-*sn*-glycero-3-phospho-ethanolamine (DOPE, Avanti Polar Lipid Inc.) and 15% polyethylene glycol C8 (PEG$_{2000}$C$_8$CER, Northern Lipid Inc., Vancouver, BC) were used at 10 mg/ml concentrations. The lipid film was formed at 50°C using a rotaevaporator (Buchi Rotavapor R110, Brinkman, Rexdale, Ont.), and then incubated at 50°C for 2 h under vacuum. The lipid film was reconstituted with distilled water and 1 M β-octylglucanopyranoside (OGP, Sigma, Mississauga, Ont.) detergent at 20% of the total preparation volume. The plasmid DNA was next added to the lipid film at a concentration of 400 μg DNA/ml of 10 mg/ml. The reconstituted preparation was transferred into dialysis tubing (Spectra/Por, MWCO: 12–14 000, Spectrum Laboratories Inc., Rancho Dominguez, CA) and dialyzed in 1 × HEPES buffer solution (150 mM NaCl, 20 mM HEPES, pH 7.4) at 23°C for 15 h. The free, non-encapsulated DNA was removed from encapsulated DNA on a DEAE Sepharose CL-6B (Sigma) anion exchange column. Encapsulation recovery ranged from 38.0 to 57.0% (data not shown). The liposome preparations were concentrated using Aquacide II (Calbiochem, La Jolla, CA) and polyethylene glycol MW 10 000 (Sigma) and then dialyzed in 1 × HEPES for an additional 2 h at 23°C. Particle size analysis of liposome encapsulated DNA was performed using a Zetasizer 3000 (Malvern Instruments, Southborough, WA).

2.3. DNA vaccination of mice

Six-week-old female BALB/c mice were obtained from the mouse breeding colony at Defence Research Establishment Suffield (DRES). The use of these animals was reviewed and approved by DRES Animal Care Committee. Care and handling of these animals followed guidelines set out by the Canadian Council on Animal Care.

Mice were immunized with naked or liposome-encapsulated plasmid DNA using intramuscular (i.m.) or intranasal (i.n.) routes of administration. For i.m. injection, mice were anesthetized with ketamine/xylazine (50

mg/kg: 50 mg/kg body weight) into the hind leg. A small incision was made exposing the quadricep muscle, and 50 μl of 1 mg/ml DNA preparation was injected slowly. The incision was then sutured. For i.n. administration, mice were anesthetized with sodium pentobarbital (50 mg/kg body wt.) by i.p. injection. When the animals were completely unconscious, 50 μl of 0.4 mg/ml DNA preparations were administered gently into one of the nostrils with a micropipettor. To avoid swallowing of the plasmid into the stomach, the i.n. dosing was given when the animals were completely anesthetized. The applied volume was naturally inhaled into the lungs. Both i.m. and i.n. groups received 1–3 additional boosts of DNA, given 4 weeks apart. One week after each boost, approximately 200 μl of blood was collected via tail bleed and analyzed for anti-HA IgA by enzyme-linked immunosorbent assay (ELISA).

2.4. Virus challenge of immunized mice

For animal virus challenge studies, a mouse-adapted strain of influenza A/PR/8/34 (H1N1) was used. This strain was obtained by at least four blind passages in mice using egg-propagated virus (ATCC, Manassas, VA) as the initial inoculum. The passaging and propagation of this mouse-adapted strain of influenza virus had been previously described in detail [11].

For the vaccine efficacy study, mice immunized with the DNA vaccine were challenge with the virus as described below. A week following the last booster dose, the animals were anesthetized with sodium pentobarbital (50 mg/kg body wt., i.p.). When the mice were completely anesthetized, they were inoculated with 50 μl of the egg-propagated virus by i.n. instillation into the nostrils. The challenge infectious dose was 5 LD$_{50}$ unless otherwise stated. At 14 days post infection, the number of surviving mice in each of the control and test groups was recorded. The number of mice in each control and test group used was 10 per group.

2.5. HA indirect ELISA

Mouse-adapted, egg-propagated influenza virus A/PR/8/34 was purified from allantoic fluid by sucrose gradient purification method. Briefly, the influenza virus was precipitated from allantoic fluid with 7% polyethylene glycol and 2.3% sodium chloride with gentle stirring for 15 h at 4°C. The virus particles were collected by centrifugation at 10 000 × g for 30 min at 23°C. The pellet was resuspended in phosphate buffered saline (PBS) and layered onto a 20–60% sucrose gradient. After ultracentrifugation at 100 000 × g for 4 h at 4°C, the virus band was isolated and dialyzed in 0.9% saline for at least 3 h at 23°C. Purified influenza virus was assayed by titration with monoclonal anti-influenza virus type A (HA) antibody (Biodesign International,

Saco, ME) to determine the optimum antigen concentration for ELISA. Dilution of 1/20 of the purified influenza virus antigen and coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 0.02% (w/v) sodium azide, pH 9.6), were used to coat the Nunc Maxisorb flat bottomed 96-well plates (Gibco BRL, Gaithersburg, MD). The plates were sealed and incubated at 4°C for 15 h. The plates were washed five times with 0.1% BSA, 1% Tween 20 in PBS, blocked with of 2% BSA, 1% Tween 20 in PBS for 1 h at 37°C and incubated with serial dilutions of test mouse sera. After 1 h incubation at 37°C and washing as described above, the bound antibody was detected by peroxidase-labeled goat anti-mouse IgA (KPL, Gaithersburg, MD). The peroxidase activity was measured using 2,2′-azino-di[3-ethyl-benzthiazoline sulfonate] (KPL) as a substrate and determined at 405 nm after 20 min of incubation at 23°C.

2.6. Statistical analysis

The survival rates of the test vaccinated and control groups were compared by the Mann–Whitney unpaired non-parametric one-tailed test (In Stat, Graph-Pad software, San Diego, CA).

3. Results

3.1. Cloning and expression of HA gene

The HA gene used in this study was originally cloned and expressed in the pT76 vector as described previously [7,8]. The HA was amplified by polymerase chain reaction, and the PCR product digested with XbaI and NotI, and the fragments were cloned in pCI vector using T$_4$ DNA ligase. The resultant construct, referred as pCI-HA10, is shown in Fig. 1.

The pCI-HA10 plasmid was transformed into competent E. coli DH5α cells. In vitro transcription/translation of the HA product was performed in the presence of canine microsomal membranes and [^{35}S]methionine, analyzed by SDS-PAGE and autoradiography (Fig. 2). The two bands of 82 and 66 K may represent the glycosylated and unglycosylated forms of HA protein. The expression of HA by pCI-HA10 was then confirmed by Western blot using a specific anti-H1 hemagglutinin monoclonal antibody (results not shown).

3.2. Efficacy of DNA vaccination

The efficacy of naked and liposome-encapsulated pCI-HA10 to protect animals against lethal challenge of influenza virus by i.n. and i.m. administrations is shown in Figs. 3 and 4. Non-immunized mice succumbed to the influenza infection at early as 7 days

post infection, and all animals were dead by day 10. All mice which received i.n. immunization with naked un-encapsulated pCI-HA10 also succumbed to the infection, with no increase in survival rate nor survival time (Fig. 3). In contrast, mice immunized intranasally with liposome-encapsulated pCI-HA10 were found to be completely protected with 100% survival rate ($P < 0.01$ vs. control or naked pCI-HA10 group).

When the pCI-HA10 DNA was administered by i.m. injection, both liposome-encapsulated and naked pCI-HA10 plasmid were shown to provide complete protection against the virus challenge (Fig. 4). In contrast, liposome-encapsulated pCI without the HA insert provided little or no protection.

3.3. IgA titers in sera

Specific IgA titers in serum samples of mice in the various immunized groups were determined by indirect ELISA assay. Mice immunized with liposome-encapsulated pCI-HA10 by intranasal route were found to contain high titers of specific IgA in the sera, while those immunized with naked unencapsulated pCI-

HA10 produced only marginally detected levels (Fig. 5). Specific IgA antibody was not detected in the serum samples from non-immunized mice or from mice immunized with liposome-encapsulated pCI without the HA insert.

Immunization of mice using i.m. injection of naked or liposome-encapsulated pCI-HA10 did not result in any significantly high levels of specific HA IgA. Up to three booster injections were given intramuscularly, but no increase in IgA levels was observed (results not shown).

4. Discussion

Genetic vaccination is a promising and exciting means of inducing both humoral and cellular protective immunity in vivo. One of the most attractive features of genetic vaccination is the possibility and flexibility to clone one or more uniquely designed gene sequences of protective antigens into the plasmids. This allows for rational designs of plasmid DNAs to be used in vaccination program against the current strains and subtypes
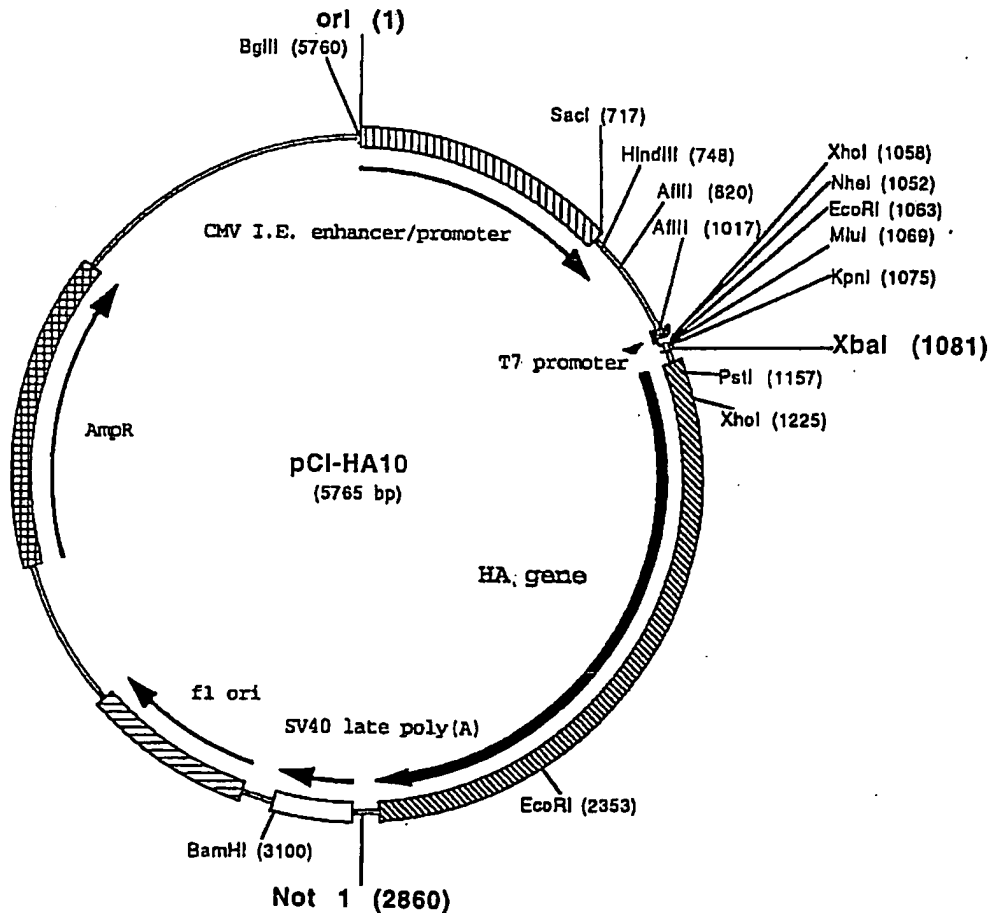

Fig. 1. Schematic representation of pCI-HA10 depicting map of plasmid, cloning and restriction sites and location of HA insert.
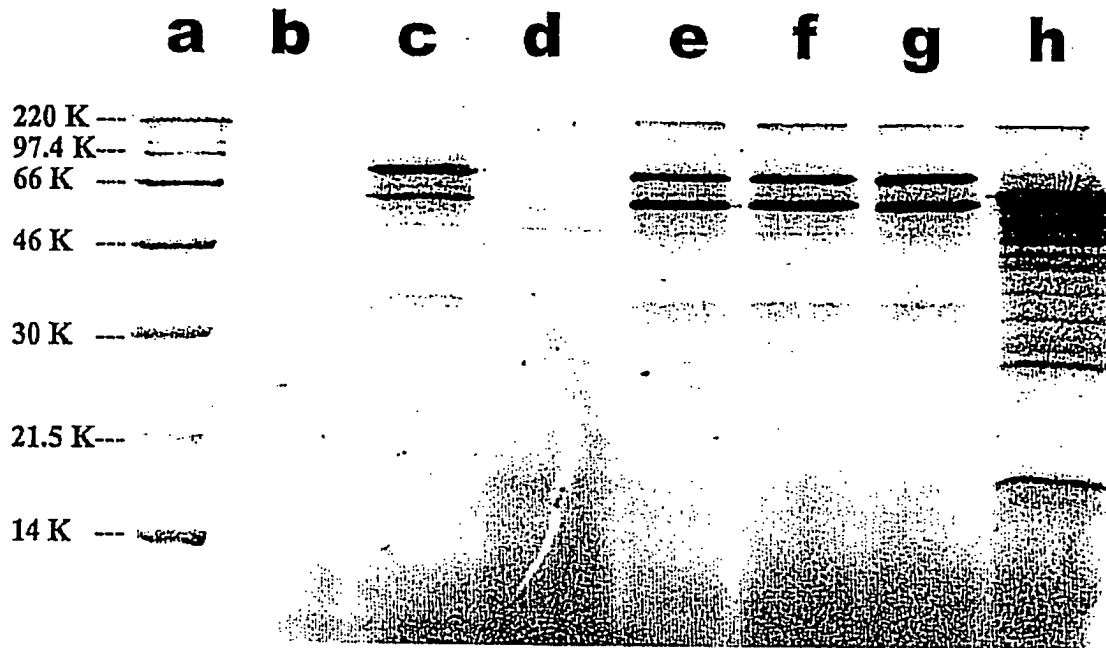
Fig. 2. Autoradiograph of HA product by SDS-PAGE. The pCI-HA10 plasmid was transformed by competent *E. coli* DH5α cells. In vitro transcription/translation of the HA product was performed in the presence of canine microsomal membranes and [^{35}S]methionine, and analyzed by SDS-PAGE and autoradiography. Lane (a) molecular weight rainbow marker, (b) no DNA, (c) positive control pT75-HA16, (d) pCI vector, no insert, (e–g) pCI-HA10, (h) luciferase translation kit control.

of influenza viruses. Due to the ease of specific gene designs and scale-up procedures afforded by molecular biology, DNA vaccination offers many advantages over conventional live, killed or attenuated vaccines such as stability, safety and design. Due to these promising attributes, development of genetic vaccines against influenza is rational, practical and valuable.

One of the important unresolved issues surrounding DNA immunization is whether the delivery of plasmid DNA by carrier systems is required for induction of protective immunity. There is ample evidence in the literature which shows that naked plasmid DNA injected directly into the muscles of animals can induce both humoral and cellular immune responses against the encoded antigens [4–6,12], although the mechanisms are not completely elucidated. These studies have shown that naked plasmid DNA are taken up and expressed by cells in vivo. Furthermore, studies by Ulmer, Liu and colleagues suggested that i.m. injection of naked plasmid DNA containing the HA can indeed induce both cellular and humoral immune responses to influenza virus [4–6]. These studies suggest that the use of a carrier system is not required for the induction of protective immunity.

Results from our present studies suggest the use of vaccine carriers such as liposomes can result in many significant advantages. When i.n. immunization of animals was carried out using pCI-HA10 encapsulated in liposomes, there was a significant enhancement in vac-

cine efficacy as well as induction of strong mucosal immunity against the expressed gene. Intranasal immunization using naked unencapsulated pCI-HA10 did not provide any significant protection, and did not result in the induction of mucosal immunity. Although naked DNA administered by i.m. injection can induce strong systemic cellular and humoral immune re-
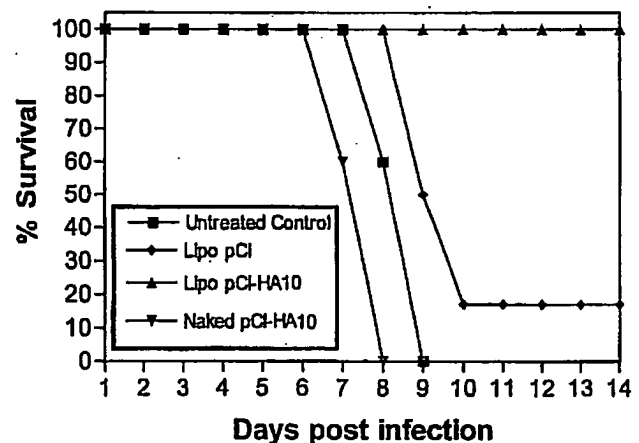

Days post infection

Fig. 3. The efficacy of intranasally administered liposome-encapsulated pCI-HA10 against influenza virus in mice. Mice intranasally immunized with one primary and three booster doses of liposome-encapsulated pCI-HA10 (Lipo pCI-HA10), naked pCI-HA10 or liposome-encapsulated pCI. At 1 week post final immunization boost, the mice were intranasally challenged with 5 LD$_{50}$ of virus. The survival rates were monitored daily.

[7] Townsend JR, McMichael AJ, Carter NP, Huddleston JA, Brown-
lee GG. Cytotoxic T cell recognition of the influenza nucleoprotein
and hemagglutinin expressed in transfected mouse L cells. Cell
1984;39:13–25.

[8] Tabor S, Richardson CC. A bacteriophage T7 RNA polymerase/
promoter system for controlled exclusive expression of specific
genes. Proc Natl Acad Sci USA 1985;82:1074–8.

[9] Long MC, Jager S, Mah DCW, JeBailey L, Mah MA, Masri SA,
Nagata LP. Construction and characterization of a novel recom-
binant single-chain variable fragment antibody against western
equine encephalitis virus. Hybridoma 2000;19:1–13.

[10] Wheeler JJ, Palmer L, Ossanlou M, MacLachlan T, Graham RW,
Zhang YP, Scherrer P, Cullis PR. Stabilized plasmid-lipid parti-
cles: construction and characterization. Gene Ther 1999;6:271–81.

[11] Wong JP, Stadnyk LL, Saravolac EG. Enhanced protection
against respiratory influenza A infection by liposome-encapsu-
lated antibody. Immunology 1994;81:280–4.

[12] McCluskie MJ, Davies HL. Novel strategies using DNA for the
induction of mucosal immunity. Crit Rev Immunol 1999;19:303–
29.

[13] Mahato RI, Anwer K, Tagliaferri F, Meaney C, Leonard P,
Wadhwa MS, Logan M, French M, Rolland A. Biodistribution
and gene expression of lipid/plasmid complexes after systemic
administration. Hum Gene Ther 1998;9:2083–99.